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14. ABSTRACT

Breast cancer is one of the leading causes of death for women in the United States and estrogen exposure has been implicated in the development of this cancer. Our lab is studying the ACI rat, an estrogen-induced breast cancer animal model, to begin to elucidate the role of estrogen in breast cancer. The ACI rat develops mammary cancer after prolonged exposure to 17β-estradiol, while the BN and genetically related COP rats do not. We have mapped a QTL conferring susceptibility to estrogen-induced mammary cancer on rat chromosome 5, for which the p16INK4A gene is a positional candidate. We found no differences in Cdkn2a gene expression between the COP, ACI and BN strains; however, gene expression was significantly elevated in the tumors relative to normal ACI mammary tissue. Methylation status of the promoter region was examined and no significant differences were found between tumor and normal tissue, suggesting that an alternative mechanism to loss of methylation accounts for upregulation of Cdkn2a gene expression in ACI mammary tumors. Sequencing of the p16INK4A gene using spleen cDNA revealed no polymorphisms in untreated ACI, COP or BN rats. In contrast, both tumors and hyperplastic mammary tissue from ACI rats treated with estrogen for 28 weeks revealed a number of independently arising mutations and polymorphisms. We will sequence genomic DNA isolated from the same tumors and mammary tissue to confirm whether the intratumoral heterogeneity is at the genomic DNA or RNA level.

15. SUBJECT TERMS
Hypermethylation, P16INK4A, CDKN2A, Tumor Suppressor, Estrogen, Mammary Cancer, Epigenetics

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Introduction. Upon chronic treatment with estrogen (E2), the ACI rat is uniquely susceptible to mammary carcinogenesis, whereas the BN and genetically related COP strains are relatively resistant. Genomewide linkage analyses of ACI x COP and ACI x BN intercrosses revealed a locus on rat chromosome 5 (RNO5) that confers susceptibility to E2-induced mammary cancer. The *p16cdkn2a* gene is a positional and functional candidate for the RNO5 locus. This gene encodes a protein with tumor suppressor activity which functions by inhibiting CDK4 and CDK6, thus regulating the G_1/S transition of the cell cycle (1). Loss of *p16cdkn2a* expression has been shown to occur in human breast cancer and is most commonly reported to be due to hypermethylation (2). We previously demonstrated by immunohistochemistry downregulation of p16 at the protein level, occuring at an early stage of E2-induced mammary carcinogenesis. We hypothesized that *p16cdkn2a* would also be downregulated in expression at the mRNA level following E2 exposure in the ACI mammary gland. To address this hypothesis we examined *p16* mRNA expression in tumors relative to normal mammary gland taken from the contralateral side of the tumor in the same ACI rat. We also wanted to address whether an epigenetic mechanism, such as the previously reported gene methylation, or a genetic mechanism could account for differences in gene expression between strains and/or during the tumorigenic process. To examine the epigenetic mechanism we performed bisulfite sequencing of the *p16* promoter to ascertain methylation status. To determine whether a genetic difference existed between the susceptible ACI and resistant COP and BN strains we sequenced the *p16* transcript and promoter region. A functional genetic variant could account for linkage of mammary cancer susceptibility to RNO5.

Body.

Task 3 (Sequencing of Cdkn2a promoter in ACI, COP and BN rats) and Alternative Task 3 (Cdkn2a mRNA sequence in tumor and normal mammary tissue of E2-treated ACI rats). As stated in Task 3 in the statement of work, both the 5'UTR and the promoter region of the *p16Cdkn2a* gene was sequenced in the ACI/SegHsd, BN/ssNHsd and COP rat strains. A total of 1kb upstream of the ATG translation start site was examined using genomic DNA isolated from mammary glands of untreated rats. Three overlapping primer sets were utilized to PCR amplify the *Cdkn2a* promoter. The same primers were utilized to directly sequence the purified PCR products. Within this 1kb interval two polymorphic regions were identified between the ACI, COP and BN strains. Both polymorphisms involved differing lengths of polypyrimidine tracts (see Table 1).

Table 1. *Cdkn2a* 5'UTR and promoter polymorphisms in ACI, COP and BN rat strains.

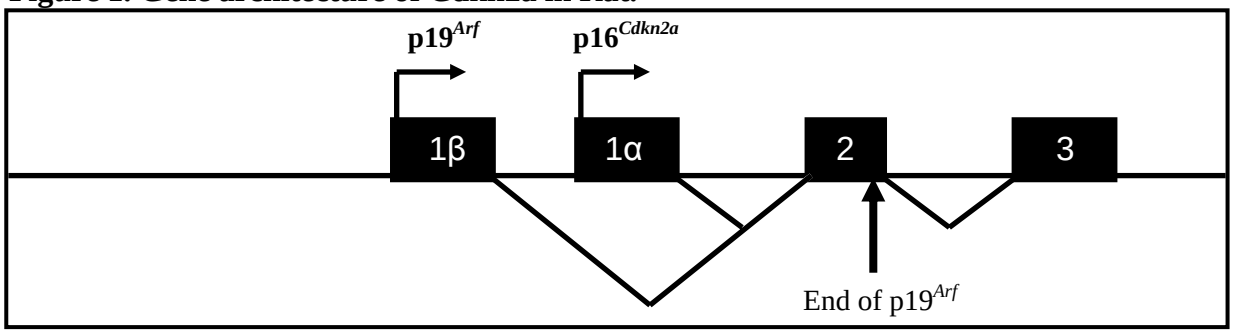
Strain	Polymorphism 1	Location*	Polymorphism 2	Location*
ACI	10 Cs	-111 → -120	41 Ts	-552 → -592
COP	10 Cs	-111 → -120	44 Ts	-552 → -595
BN	13 Cs	-111 → -123	30 Ts	-552 → -581

*Location gives the bp relative to the ATG translation start site (+1).

The sequences for the *p16Cdkn2a* promoter and 5'UTR were deposited in GenBank under the following accession numbers: DQ350895 (ACI), DQ350896 (COP), and DQ350894 (BN). There are a number of known transcription factor binding sites that have been shown to regulate the p16 promoter in mouse and human. These sites include Ets1 (3), Sp1 (4, 5), RBAR1 (6), RNA helicase A (7), INK4a transcription silence element (8), and E47 (9, 10). However, neither polymorphic region detected in our sequence data affect any of these known elements. It remains to be shown whether these polypyrimidine tract length polymorphisms induce a functional change at the *p16Cdkn2a* promoter.

The *Cdkn2a* gene encodes two separate proteins, both p16 and p19Arf, which share exons 2 and 3, but each having unique first exons and promoters (see Figure 1). Exons 1α and 1β each join exon 2 in an alternate reading frame leading to two entirely unique proteins. While sequencing the upstream region of *p16Cdkn2a* gaps were encountered. Additionally, exon 1β sequence for p19Arf had not been previously reported in the rat. Therefore, we sequenced through these gaps and obtained sequence data for exon 1β in all three strains. These sequences were also deposited in GenBank under the following accession numbers: DQ336095 (ACI), DQ336096 (COP), and DQ336094 (BN).

Figure 1. Gene architecture of Cdkn2a in Rat.



In Alternative Task 3 we also proposed to determine the mRNA sequence of *Cdkn2a*. We first determined the mRNA sequences for all 3 strains in untreated rats. For p19Arf we utilized lung tissue and for p16 we utilized spleen. Total RNA was isolated from the respective tissue and first strand cDNA synthesis was done using Oligo(dT) priming. Two separate primer sets were used to amplify the p19 and p16 mRNAs. No strain differences were identified in the coding regions of either p19Arf or p16. These sequences were also deposited in GenBank. For p19Arf the accession numbers were: AY679727 (BN), AF474974 (ACI) and AF474975 (COP). For p16 the accession numbers were: AY679728 (BN), AF474976 (ACI) and AF474977 (COP). Assuming the promoter variants do not result in a functional change in gene expression between strains, taken together with a lack of polymorphisms within the *Cdkn2a* coding region, this suggests that this gene does not account for the linkage of our mammary cancer phenotype to RNO5 and places a lower priority on this gene as a positional candidate. Although, epigenetic mechanisms affecting the function of this gene could still play a role in the progression of these tumors.

We also examined the possibility of somatic mutation of *Cdkn2a* in E2-induced mammary cancers. To do this we isolated total RNA from 8 tumors within 6 ACI rats. We also isolated total RNA from normal mammary gland, taken from the contralateral side of the tumor in the same 6 ACI rats. First strand cDNA synthesis was done with Oligo(d)T priming from which the *p16Cdkn2a* transcript was PCR amplified. TA cloning of purified PCR products was done and a total of 10 clones for each tumor sample and 5 clones for each normal sample were sequenced. These ACI animals were treated with E2 for 28 weeks, with the resulting tumors histologically being characterized by ductal carcinoma *in situ* (DCIS). Thus, the grossly normal tissue taken from these rats likely also contain focal regions of atypical hyperplasia. The *p16Cdkn2a* sequencing results from tumor and grossly normal mammary tissue are appended in supplemental Tables 1 and 2. Despite a lack of mutations at the genomic level in untreated ACI rats, there were a large number of somatic mutations detected in ACI mammary gland of 28 week E2-treated rats. Somatic mutation was evident both in the grossly normal and tumor tissue, suggesting a mechanism occuring in precursor lesions to DCIS. In fact, it appears that there was a higher degree of mutation heterogeneity in the normal compared to tumor tissue. The intratumoral heterogeneity was a surprising result and we will confirm these findings by sequencing *p16Cdkn2a* using genomic DNA isolated from mammary tissue of the same ACI animals. Although a mechanism for this heterogeneity has not previously been reported, these findings are consistent with other recent findings in the literature. In colorectal cancer (CRC) progression there is a reduction in intratumoral heterogeneity from early to advanced stages (11). Further, the majority of Ras mutations found in these colorectal tumors were transitions (12). We found that >80% of the *Cdkn2a* mutations were A/G or C/T transitions. We will continue these studies to determine at what time point these somatic mutations arise and attempt to correlate this with grade of histologic lesion. We will also sequence from genomic DNA to ensure that these transitions are at the genomic level and not post-transcriptional. Further, we will sequence additional genes on chromosome 5 to assess whether this somatic hypermutability is locus specific or whether there are other loci impacted and a more global deregulation is involved. Comparative genomic hybridization (CGH) data from these ACI tumors frequently show a loss of chromosome 5.

Task 2 (Determine the mRNA expression of p16Cdkn2a in the ACI rat). *P16Cdkn2a* mRNA expression profiles were generated for a battery of 28 week E2-treated rats, utilizing total RNA isolated from both tumor and grossly normal mammary tissue in the same rat for comparison. QRT-PCR was performed by TaqMan assay on an ABI 7000. Relative ng of p16 expression were computed using spleen cDNA as the standard and normalizing raw data to GAPDH gene expression. Figure 2 shows that the level of p16 expression was variable between biologic replicates, however in general, p16 expression was typically elevated in tumors relative to the grossly normal tissue.

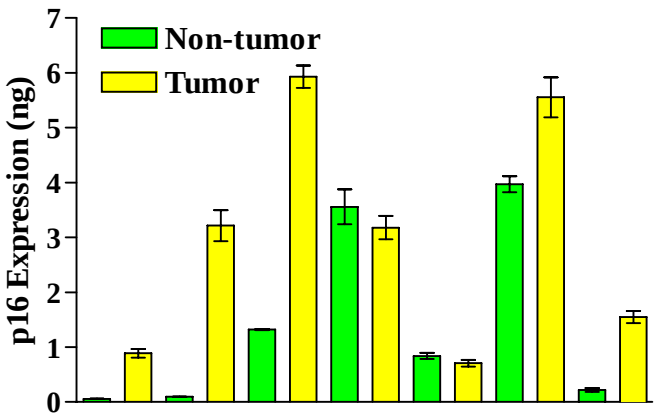


Figure 2. mRNA expression of *p16Cdkn2a* in tumor and grossly normal mammary tissue from 28 week E2-treated ACI rats. QRT-PCR was done by TaqMan assay. Raw data were normalized by GAPDH expression as an endogenous control. Relative quantitation was computed using a spleen cDNA standard curve. Shown are pairs of tumor and grossly normal tissue from each of 7 ACI rats.

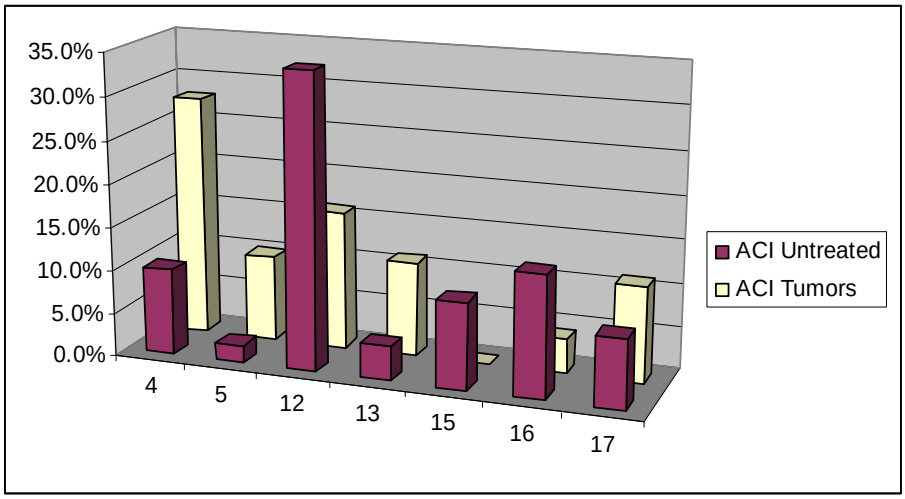
The finding of p16 upregulation in tumors relative to grossly normal tissue is counter to what was expected *a priori*. We postulated that *p16Cdkn2a*, which encodes a protein with tumor suppressor activity, would be downregulated in tumors relative to normal mammary tissue. This contradictory finding, however, is consistent with some of the more recent literature in which p16 is in fact upregulated at the mRNA level in tumors (14, 15). In future studies we will perform Western blot analysis to determine if the mRNA upregulation correlates at the protein level in these same samples.

Task 4 (Determine the Cdkn2a methylation status in the ACI rat). The methylation status of the promoter and 5'UTR of the *p16Cdkn2a* gene was assessed in ACI tumors compared with normal ACI mammary gland. DNA was isolated using Trizol (Gibco-Brl). A total of 10 tumors from 6 E2-treated rats were utilized, as well as DNA isolated from normal mammary gland of 5 untreated ACI rats. Ten independent clones were sequenced for each of the 10 tumor and 5 normal mammary DNA samples. DNA (1.35μg) was bisulfite treated using the EZ DNA Methylation Kit (Zymo Research). Primers obtained from Abe et al. (16) were used to amplify two overlapping portions of the *p16Cdkn2a* promoter region, including the putative transcription start site and known transcription factor binding sites. As a positive control for methylation, DNA was treated with the enzyme SssI Methylase (Promega), which methylates all CpG sites. As a negative control, DNA from spleen tissue from each of the rats was used.

Twenty-two separate CpG sites were examined by bisulfite sequencing. For each sample the percentage of clones with methylation at a given site was computed. These percent methylations were then averaged across all samples for a given site. Fifteen of the 22 sites showed average percent methylation below 10% in both the tumor and normal samples. These were not further examined. Seven of the sites showed greater than 10% average methylation in tumor and/or normal samples. The methylation status of these sites are summarized in Figure 3. Sites 4, 5 and 13 showed higher average methylation in the tumor relative to normal samples. In contrast, sites 12, 15 and 16 exhibited greater methylation in the normal relative to tumor samples. Site 17 showed fairly equivalent methylation status in both groups. Although there were some differences between tumor and normal samples, the relative fraction of methylated clones was a small minority, suggesting that methylation likely does not account for the expression differences between normal and tumor tissue. Since we found an upregulation of p16 mRNA expression in tumors relative to normal tissue, we would expect a loss of methylation in the tumors. Our results, which show a small number of methylated clones and a high degree of

variance, suggest that methylation does not play a significant regulatory role in gene expression in these tumors, despite evidence that this epigenetic mechanism does play a role in some human tumors.

Figure 3. Summary of methylated sites in the *p16Cdkn2a* promoter. Shown is the average percent methylation in tumor vs. untreated samples at 7 CpG sites in the *p16Cdkn2a* promoter.



Key Research Accomplishments

- Sequenced 1kb of the 5' UTR and promoter region of the *p16Cdkn2a* gene in 3 strains of rats
  - Identified 2 length polymorphisms between the ACI, COP and BN strains
  - Compared rat sequence to mouse and human
  - Sequenced through gaps in the promoter region and identified the rat sequence for exon 1β of the *p19Arf* gene
- Sequenced *p16Cdkn2a* mRNA from untreated and E2-treated rats
  - Untreated ACI, COP and BN rats showed no polymorphisms in *p16Cdkn2a* indicating no strain differences at the genomic level
  - 28 week E2-treated ACI rats showed somatic hypermutation of *p16Cdkn2a* both in tumors and in grossly normal tissue
  - Significant intratumoral heterogeneity was demonstrated for these ACI mammary tumors
- Quantitative RT-PCR revealed upregulation of *p16Cdkn2a* mRNA expression in ACI tumor relative to nontumor mammary tissue
- Bisulfite sequencing of the *p16Cdkn2a* 5'UTR and promoter revealed no significant differences in the methylation status of tumor and nontumor samples

Reportable Outcomes

1. Genbank Submissions
   - Accession No. DQ336096. Rattus norvegicus strain COP/CrCrl cyclin-dependent kinase inhibitor 2a p19Arf (Cdkn2a) gene, exon 1 beta and partial cds
   - Accession No. DQ 336095. Rattus norvegicus strain ACI/SegHsd cyclin-dependent kinase inhibitor 2a p19Arf (Cdkn2a) gene, exon 1 beta and partial cds
   - Accession No. DQ 336094. Rattus norvegicus strain BN/SsNHsd cyclin-dependent kinase inhibitor 2a p19Arf (Cdkn2a) gene, exon 1 beta and partial cds
   - Accession No. DQ350896. Rattus norvegicus strain COP/CrCrl p16 (Cdkn2a) gene, promoter region and 5'UTR

- Accession No. DQ350895. Rattus norvegicus strain ACI/SegHsd p16 (Cdkn2a) gene, promoter region and 5'UTR
- Accession No. DQ350894. Rattus norvegicus strain BN/SsNHsd p16 (Cdkn2a) gene, promoter region and 5'UTR
- Accession No. AY679728. Rattus norvegicus strain BN/SsNHsd cyclin-dependent kinase inhibitor 2a p16Ink4a (Cdkn2a) mRNA, complete cds
- Accession No. AF474977. Rattus norvegicus strain COP cyclin-dependent kinase inhibitor 2a p16Ink4a (Cdkn2a) mRNA, complete cds
- Accession No. AF474976. Rattus norvegicus strain ACI/SegHsd cyclin-dependent kinase inhibitor 2a p16Ink4a (Cdkn2a) mRNA, complete cds
- Accession No. AY679727. Rattus norvegicus strain BN/SsNHsd cyclin-dependent kinase inhibitor 2a p19Arf (Cdkn2a) mRNA, complete cds
- Accession No. AF474975. Rattus norvegicus strain COP cyclin-dependent kinase inhibitor 2a p19Arf (Cdkn2a) mRNA, complete cds
- Accession No. AF474974. Rattus norvegicus strain ACI/SegHsd cyclin-dependent kinase inhibitor 2a p19Arf (Cdkn2a) mRNA, complete cds

2. Abstracts
   - Huerta, JA and JD Shull. 2005. Evaluation of *p16Cdkn2a* promoter methylation during estrogen-induced mammary carcinogenesis in the ACI rat. Cold Spring Harbor –Rat Genomics and Models Meeting.
   - Huerta, JA, BS Schaffer, R Streblow, B Xie, and JD Shull. 2006. Evaluation of *p16Cdkn2a* promoter methylation during 17β-estradiol-induced mammary carcinogenesis in the ACI rat. American Association for Cancer Research 2006 Meeting.
   - Deffenbacher, KE, KL Pennington, S Kurz, T Strecker, K Hansen, and JD Shull. 2005. Impact of hormonal and genetic factors on gene expression profiles in the anterior pituitary gland of the rat. Cold Spring Harbor –Rat Genomics and Models Meeting. Platform Presentation.
   - Kurz, SG, K Hansen, BS Schaffer, TE Strecker, KL Pennington, V Shivaswamy, KE Deffenbacher and JD Shull. 2005. Development and characterization of the ACI.COP-Ept2 congenic strain reveals tissue specific actions of Ept2 as a genetic determinant of responsiveness to estrogens. Cold Spring Harbor –Rat Genomics and Models Meeting. Platform Presentation.
   - Deffenbacher, KE, KL Pennington, SG Kurz, TE Strecker, and JD Shull. 2006. Impact of Hormonal and Genetic Factors on Gene Expression Profiles in the Anterior Pituitary Gland of the Rat. American Association for Cancer Research –Pathobiology of Cancer Workshop.

Conclusions

The work done in this proposal examined both genetic and epigenetic mechanisms by which *p16Cdkn2a* gene function might be altered in E2-induced mammary carcinogenesis in the ACI rat. The *p16* promoter is found to be methylated and shut down in some human mammary tumors. We examined the methylation status of the rat *p16* promoter in ACI tumors relative to grossly normal mammary tissue. We found no significant differences in methylation status, suggesting that this mechanism of *p16* gene regulation does not occur in ACI mammary tumors. We also examined the mRNA expression of *p16* in tumors relative to normal tissue. Since *p16* encodes a protein with tumor suppressor activity, we assumed *a priori* that p16 gene expression might be lost in tumors. In fact, *p16* gene expression was consistently found to be upregulated in the tumors relative to normal tissue. More recent literature corroborates this finding. In a number of hormone-dependent cancers *p16* gene expression is found upregulated. Mechanistically, it is unknown whether this serves an attempted compensatory role and simply the p16 pathway is overridden by other factors. Regardless of function, this overexpression may serve as a biomarker for a specific stage of tumor progression. In future studies we will examine

expression at different time points to determine when this upregulation occurs and how it correlates with histologic grade. We will also perform Western blot analysis on the corresponding tissues to ascertain whether mRNA expression level correlates with protein level.

Since the epigenetic mechanism of methylation does not appear to account for differences in gene expression between normal and tumor tissue, we also looked at whether there were underlying genetic differences between the tumor susceptible ACI and resistant COP and BN strains. A genetic variant conferring functional and/or expression differences between the strains could account for the linkage of our mammary phenotypes to RNO5. To address this, we sequenced the entire coding region, 5'UTR and promoter region of the *p16Cdkn2a* gene in untreated ACI, COP and BN rats. These sequences were deposited in GenBank (see reportable outcomes section). There were no coding mutations between strains at the genomic level. Within the 1kb upstream sequence of the promoter, 2 polypyrimidine tract length polymorphisms were identified between the 3 rat strains. The *p16* promoter is very well functionally characterized in mouse and human, and these 2 polymorphisms do not impact any of the known transcription factor binding sites or regulatory sequences. Reporter assays using primary cells or a mammary cell line could identify whether these 2 polymorphisms impact gene expression. The more likely scenario is that some trans regulatory factor generates the differential regulation of this gene in tumor relative to normal tissue. In contrast to the genomic results, there were a large number of independently arising somatic mutations in the coding region of *p16*. This somatic hypermutation occurred in both the grossly normal and tumor tissues of 28 week E2-treated ACI rats. We will sequence other loci in the tumors to see if this event is locus specific or if there is global infidelity in the tumors. Initial CGH analyses of these tumors indicate chromosomal instability. The finding of intratumoral heterogeneity is novel and we will conduct future experiments to identify a potential mechanism.

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Appendix: Supplemental Data

Supplemental Table 1. *p16Cdkn2a* coding mutations identified in mammary gland tumors in ACI rats.

Sample	Fraction[a]	Location[b]	Nucleotide change	Codon change	AA change
5765A	2/10	+353	T-->C	GTC-->GCC	**Val-->Ala**
	1/10	+421	T-->C	TCT-->CCT	**Ser-->Pro**
	1/10	+133	A-->G	ATG-->GTG	**Met-->Val**
16C	1/11	+211	T-->C	TCC-->CCC	**Ser-->Pro**
	1/11	+311	G-->T	CGC-->CTC	**Arg-->Leu**
	1/11	+469	A-->G	AAG-->GAG	**Lys-->Glu**
	1/11	+187	A-->G	AAC-->GAC	**Asn-->Asp**
	1/11	+202	A-->G	ACC-->GCC	**Thr-->Ala**
	1/11	+209	T-->C	CTC-->CYC	**Leu-->Pro**
	1/11	+427	T-->C	CTC-->CYC	**Leu-->Pro**
	1/11	+424	T-->C	TGC-->CGC	**Cys-->Arg**
5765B	1/11	+74	A-->G	GAA-->GGA	**Glu-->Gly**
	3/11	+101	A-->G	AAC-->AGC	**Asn-->Ser**
	1/11	+260	T-->C	GTA-->GCA	**Val-->Ala**
	1/11	+62	G-->A	CGG-->CAG	**Arg-->Gln**
	1/11	+326	T-->C	CTG-->CCG	**Leu-->Pro**
15A	1/11	+20	G-->A	AGA-->AAA	**Arg-->Lys**
	1/11	+197	A-->G	GAC-->GGC	**Asp-->Gly**
	1/11	+397	T-->C	TCC-->CCC	**Ser-->Pro**
7A	1/10	+365	T-->C	TTG-->TCG	**Leu-->Ser**
	1/10	+62	G-->A	GGG-->GAG	**Gly-->Glu**
	1/10	+446	T-->C	CTA-->CCA	**Leu-->Pro**
3A	1/9	+101	A-->G	AAC-->AGC	**Asn-->Ser**
	1/9	+427	T-->A	TCA-->ACA	**Ser-->Thr**
	1/9	+460	C-->G	CAA-->GAA	**Gln-->Glu**
	1/9	+392	A-->G	AAC-->AGC	**Asn-->Ser**
5763A	1/12	+107	T-->A	TTC-->TAC	**Phe-->Tyr**
	1/12	**+2**	T-->C	ATG-->ACG	**Met-->Thr**
	1/12	+248	T-->C	CTA-->CCA	**Leu-->Pro**
5763B	1/10	+187	A-->G	AAC-->GAC	**Asn-->Asp**
	1/10	+318	G-->A	CGC-->CAC	**Arg-->His**
	1/10	+19	A-->G	AGA-->GGA	**Arg-->Gly**
	1/10	+370	T-->C	TAT-->CAT	**Tyr-->His**

a. Fraction is the number of individual clones with a given mutation
b. Location is the nucleotide position of a mutation relative to the translation start site (+1)

Supplemental Table 2. *p16Cdkn2a* coding mutations identified in grossly normal E2-treated mammary gland of ACI rats.

Sample	Fraction[a]	Location[b]	Nucleotide change	Codon change	AA change
5765N	1/5	+101	A-->G	AAC-->AGC	**Asn-->Ser**
15N	1/5	+107	T-->C	TTC-->TCC	**Phe-->Ser**
	1/5	+164	T-->C	CTG-->CCG	**Leu-->Pro**
	1/5	+404	T-->C	GTC-->GCC	**Val-->Ala**
	1/5	+436	del C	Frameshift @aa146	**Frameshift**
16N	1/5	+101	A-->G	AAC-->AGC	**Asn-->Ser**
5763N	1/7	+469	A-->G	AAG-->GAG	**Lys-->Glu**
	1/7	+352	G-->A	GTC-->ATC	**Val-->Ile**
3N	1/7	+92	A-->G	AAC-->AGC	**Asn-->Ser**
	1/7	+370	T-->C	TAT-->CAT	**Tyr-->His**
7N	6/7	+62	G-->A	CGG-->CAG	**Arg-->Gln**
	1/7	+235	C-->T	CGG-->TGG	**Arg-->Trp**
	5/7	+326	T-->C	CTG-->CCG	**Leu-->Pro**
	1/7	+395	T-->C	GTT-->GCT	**Val-->Ala**

a. Fraction is the number of individual clones with a given mutation
b. Location is the nucleotide position of a mutation relative to the translation start site (+1)